[CANCER RESEARCH 63, 2179–2187, May 1, 2003]

The *Septin* 9 *(MSF)* Gene Is Amplified and Overexpressed in Mouse Mammary Gland Adenocarcinomas and Human Breast Cancer Cell Lines[1]

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ABSTRACT

The expression of polyomavirus middle T antigen under the control of the mouse mammary tumor virus promoter in transgenic mice results in the induction of aggressive mammary gland adenocarcinomas at an early age. We screened 26 tumors for chromosomal aneuploidies using SKY and CGH. In 70% of the tumor samples we could detect high-level copy number gains, which mapped to chromosome band 11E2, a region orthologous to human 17q25.3. We then identified a bacterial artificial chromosome clone that labeled double-minute chromosomes found in the tumor metaphases. This bacterial artificial chromosome clone showed sequence homology to a member of the septin gene family. Real-time PCR analysis revealed a consistently increased expression of septin 9 (*Sept9*), not only in polyomavirus middle T antigen-induced, but in a wide variety of mouse models of breast cancer. Six of 9 human tumor cell lines also revealed elevated expression levels of *Sept9*. The family of septin genes is involved in a plethora of cellular processes, including cytokinesis in yeast and vesicle transport, and possesses GTPase activity. We identified down-regulation of *Thsp1*- and *Bax*-regulated apoptotic response in those tumors with *Sept9* overexpression, an effect that could be reversed by inhibiting *Sept9* expression using transfection with small interference RNA. Our results now suggest that signaling via members of the septin family plays a novel and common role in breast tumorigenesis.

INTRODUCTION

Mouse models of human breast cancer are used widely to study molecular pathways of tumorigenesis (1). The overexpression of oncogenes known to be involved in human breast cancer, such as *c-Myc*, *HER2/Neu*, and *Ccnd1*, with tissue-specific promoters induces tumors in the mammary gland of transgenic mice (2, 3). Likewise, the inactivation of tumor suppressor genes via introduction of viral oncogenes or by conditional knockout using CreLox technology can promote tumorigenesis (4, 5). Human breast cancers, like other epithelial cancers, are characterized by a recurrent and conserved pattern of genomic imbalances (6). These imbalances result in up- or down-regulation of oncogenes and tumor suppressor genes, respectively, located on these chromosomes or chromosomal regions. The strong conservation of specific chromosomal imbalances in human solid tumors suggests that these aneuploidies are crucial events in tumorigenesis. Therefore, it is reasonable to assume that the identification of such aneuploidies in mouse models of breast cancer and the comparison to their distribution in human tumors can contribute to the validation of respective tumor models. The detection of recurring regions of genomic imbalances also can provide entry points for the molecular cloning of cancer-associated genes. The advent of molecular cytogenetic tools has allowed the screening of the entire murine genome for such genetic alterations. In particular, CGH and SKY have facilitated the analysis of such changes in mouse tumors (7, 8). We used these techniques previously to characterize genomic imbalances in several models of human breast cancer. For example, the overexpression of *c-Myc* or *HER2/Neu* oncogenes under MMTV[4] or endogenous promoters results in tumorigenesis; however, cytogenetic analysis revealed that secondary genomic alterations are required, most notably deletions that map to mouse chromosome 4 (band C-E), a region that is orthologous to human 1p32–36 (9, 10). However, conditional inactivation of the breast cancer susceptibility gene *Brca1* requires amplification of terminal mouse chromosome 11, which in some cases includes the *HER2/Neu* oncogene in addition to losses on mouse chromosome 4 (4, 9).

Here we have analyzed the consequences of mammary gland-specific expression of the PyV-mT antigen on genomic stability with a particular emphasis on the development of secondary chromosomal and genetic aberrations. The expression of PyV-mT results in the formation of mammary tumors (11). Middle T antigen interacts with growth factor signaling pathways via activation of the src-tyrosine kinases, phosphatidylinositol 3′-kinase, and phospholipase C. On ShcA it creates binding sites for Grb2, of which the function is enhanced by overexpression (12). These pathways stimulate the mitogen-activated protein kinase cascade and promote cell division (13). Consequently, the mice develop multifocal mammary gland adenocarcinomas by 5 weeks of age (11). However, as in other murine models of breast cancer, it is not clear whether additional genetic aberrations are required for tumorigenesis and which molecular pathways are involved. The molecular cytogenetic and genetic characterization of 26 PyV-mT-induced mammary gland adenocarcinomas revealed a strictly conserved pattern of genomic imbalances, including high-level copy number increases that map to distal chromosome 11. The consequences of this amplification on gene expression levels were assessed using quantitative real-time PCR in this and other mouse models, and in cell lines derived from human breast carcinomas. The results suggest a novel signaling pathway involved in breast tumorigenesis.

MATERIALS AND METHODS

Tumors and Cell Lines. Mammary epithelial cell lines were derived from 15 different MMTV-PyV-mT primary tumors and 11 cell lines derived from their lung metastases. C-myc 83, brt-1, brt-5, brt-10, and pbrt-1 were derived from the MMTV-*c-Myc* transgenic model, and the *Brca1* conditional knockout as reported previously (7, 9). FF2, 4, 5, 7, 8, 9, 10, 12, and 21 cell lines were derived from the *Notch4* transgenic mice described earlier (14). Metaphase chromosomes were prepared from the cell lines at passages 15–20 after

Received 11/5/02; accepted 3/4/03.

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[1] Supplemental data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

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[4] The abbreviations used are: MMTV, mouse mammary tumor virus; PyV-mT, polyomavirus middle T antigen; BAC, bacterial artificial chromosome; FISH, fluorescence *in situ* hybridization; dmin, double min chromosome; siRNA, small interference RNA primer; GFP, green fluorescent protein; CGH, comparative genomic hybridization; sky, spectral karyotyping; Mbp, mega base pair.

Colcemid arrest (1 h; final concentration 0.1 μg/ml), and standard hypotonic treatment and fixation in methanol/acetic acid. The human breast cancer cell lines BT 549, UACC 812, Zr 75-30, UACC 893, T47D, Pc3, and MDA 157 (American Type Culture Collection, Manassas, VA), SUM159 (University of Michigan, Ann Arbor, MI), and MPE 600 (Vysis, Downers Grove, IL) were cultured as described.[5]

Molecular Cytogenetic Analysis. SKY and CGH were performed essentially as described before. Details of the protocols can be found elsewhere.[6] The following mouse BAC clones for FISH analysis were selected using information provided elsewhere:[7,8] RP23-147O23, 284L12, 101J23, 341C5, 202G21, 369E6, 382B19, 99M13, 84C12, 28I4, 354K2, 333B13, 48A17, and 480B11 (Invitrogen, Carlsbad, CA). The clones were labeled by nick-translation and hybridized to tumor metaphase chromosomes according to standard procedures. Images were acquired using a DMRXA microscope (Leica, Wetzlar, Germany), connected to a Sensys CCD camera (Roper Scientific, Tucson, AZ) using Q-FISH software (Leica Microsystems, Cambridge, United Kingdom). Complete results of the SKY and CGH analysis and the derived tumor karyotypes can be retrieved from the National Cancer Institute/National Center for Biotechnology Information SKY and CGH website.[9]

Total mRNA from the human breast cancer cell lines SUM159, BT 549, UACC 812, MPE 600, Zr 75-30, UACC 893, T47D, Pc3, and MDA 157 was extracted using Qiagen mini kit (Qiagen, Valencia, CA).

Quantitative Analysis of Gene Expression. For real-time PCR experiments, were 2 μg of total RNA reverse-transcribed from by using a first-strand cDNA synthesis kit (Invitrogen, Grand Island, NY) and analyzed with the ABI PRISM7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and carboxyfluorescein/6-carboxytetramethylrhodamine real-time PCR probes (Synthegen, Houston, TX) for the cDNA-specific real-time quantitative PCR assay were designed using Primer express 1.5 software for human *GRB2*, *MSF*, and *RAC3* genes, and for the mouse *Bax*, *Grb2*, *HER2/Neu*, *Rac3*, *Sept9*, and *Thbs1* genes (see Supplemental Table 1). The custom designed primers were used along with commercially available primers for mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA and 18S rRNA housekeeping controls (Applied Biosystems) to determine relative mRNA expression levels. cDNA derived from normal mouse brain and normal mammary glands were used for generating a standard curve and normalizing data. The normal mammary gland standard was arbitrarily designated as 1.0. cDNA from samples 143mt, 404mt, 2571mt, and pbrt-5 were hybridized to 96 gene cDNA arrays filters from the mouse cancer pathway finder and cell cycle series (SuperArray, Bethesda, MD) following the suppliers protocol.

siRNA Transfection. siRNAs corresponding to pEGFP reporter gene and to *Sept9* mRNAs were designed as recommended (15), with two base overhangs (Xeragon, Germantown, MD). The following gene-specific sequences were used successfully: si-GFP antisense 5′-GAACUUCAGGGUCAGCUUGCCG-dTT-3′; *Sept9* siRNA sense 5′-GUCCACUUUAAUCAAUACC dTT-3′ and antisense 5′-GGUAUUGAUUAAAGUGGAC dTT-3′. Annealing for duplex siRNA formation was performed in annealing buffer [100 mM potassium acetate, 30 mM HEPES-KOH at (pH 7.4), and 2 mM magnesium acetate] for 1 min at 90°C, followed by 1 h at 37°C. Transfections of reporter plasmid and siRNA were performed with Oligofectamine (Invitrogen) in six-well plates. Briefly, 3 μl of oligofectamine diluted in Opti-MEM was applied to the siRNA duplex mixture (60 pmol in 3 μl annealing buffer) and incubated 25 min at room temperature. The siRNA duplex oligofectamine mixture was added to cultured cells (40–50% confluent). The cells were seeded the previous day in tissue culture medium with 10% fetal bovine serum but without antibiotics. Transfection was carried out for 48 h. mRNA was extracted with Qiagen mini kit (Qiagen), and expression level for *Bax*, *Sept9*, and *Thsp1* was determined by real-time PCR.

RESULTS

To identify secondary genetic events required for PyV-mT-associated carcinogenesis in the mammary gland, we analyzed 26 cell lines established from primary tumors or tumor metastases by CGH and SKY. These screening tests for genomic imbalances and chromosomal translocations revealed three hotspots of genetic instability in this tumor model. We detected frequent losses of the distal part of chromosome 4 (10 of 26 cases), and copy number increases on chromosomes 11 and 15 (17 and 10 cases, respectively). The chromosome 11 gain was particularly striking, because high-level copy number increases (amplifications) were focused to just chromosome band 11E2. A summary of the genomic imbalances is presented in Fig. 1 and the results can also be viewed at the SKY/CGH website.[9] SKY analysis revealed that different chromosomal events led to the genomic amplification of chromosome 11 band E2. Nine tumors showed jumping translocations (recipient chromosomes were 1, 4, 5, 6, 7, and X), and 4 tumors showed duplications of distal chromosome 11 (Fig. 2, *A* and *B*). Two tumors (158mt and 2571mt) revealed numerous dmins, a cytogenetic feature reflecting the amplification of oncogenes in solid tumors (8–20 dmin/cell in 50% of the cells). Mouse chromosome 11 contains a plethora of breast cancer-associated genes including the tumor suppressor genes *Trp53* and *Brca1*, and the *HER2/Neu* oncogene, a member of the *Egf* receptor family. To determine whether these genes were deleted or amplified, we performed FISH analysis with gene-specific BAC clones. In each case the copy numbers were equal to the ploidy of the cell. *c-Myc*, another oncogene commonly up-regulated in breast carcinomas, was also present in the normal copy number on mouse chromosome 15 (Fig. 2, *C* and *D*). CGH analyses of human carcinomas suggested a second region of frequent amplification that maps to chromosome band 17q23 (16, 17). Candidate genes in this amplicon include *Rad51c* and *Tbx2*. Neither of these genes was involved in the copy number increases on the orthologous region of mouse chromosome 11 (Fig. 2, *E* and *F*). To create additional probes for band 11E2 we established a physical map of BAC clones specific for the cytogenetically identified region of copy number gain on chromosome 11E2. Eleven BAC clones spanning a region of 17 Mbp were selected and hybridized to tumor metaphase chromosomes derived from five different tumors representing the diversity

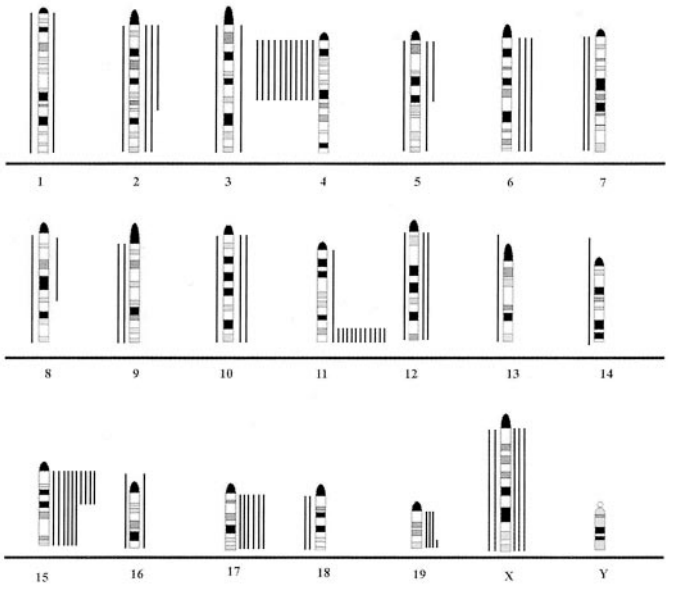

Fig. 1. Summary of genomic imbalances detected by CGH and SKY of 26 tumors induced by overexpression of PyV-mT under the control of the MMTV promoter. Genomic imbalances that result in gains are plotted on the *right* side of the ideogram, whereas losses are displayed on the *left*. Note the predilection of only few chromosomes or chromosomal bands to recurrent copy number losses (chromosome 4), or gains (chromosomes 11 and 15). A complete view of the aberrations in individual cases can be viewed elsewhere.[9]

[5] Internet address: http://www.atcc.org/.
[6] Internet address: www.riedlab.nci.nih.gov.
[7] Internet address: http://www.ncbi.nlm.nih.gov/genome/guide/mouse/index.html.
[8] Internet address: http://www.ensembl.org/Mus_musculus.
[9] Internet address: http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi.

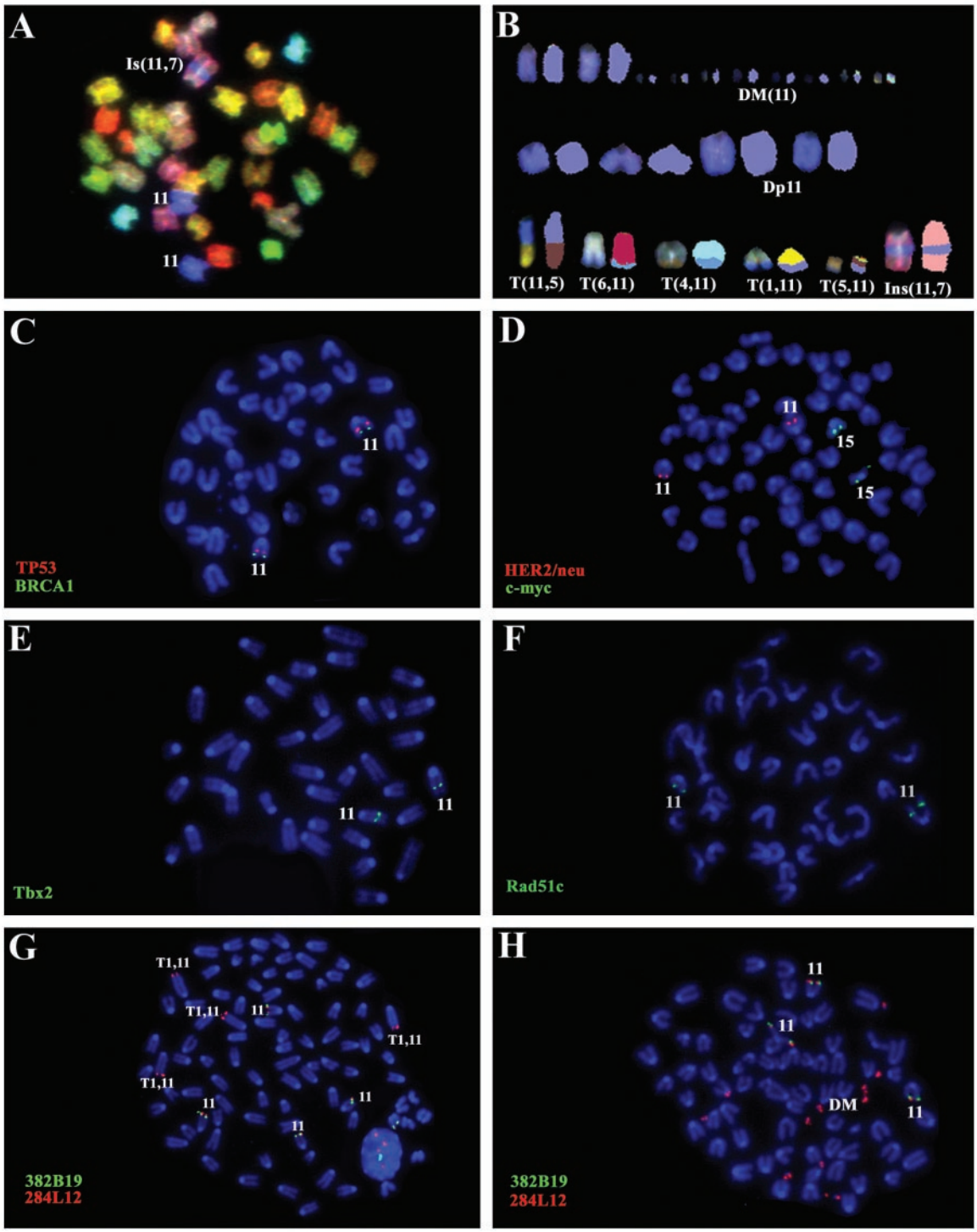

Fig. 2. Molecular cytogenetic characterization of PyV-mT induced tumors and high-resolution amplicon mapping. *A*, SKY of a representative metaphase from tumor 355mt. Note extra chromosome 11 material involved in an insertion. *B*, summary of chromosome rearrangements observed in several tumors revealed multiple possible mechanisms for the acquisition of copy number increases on chromosomes 11, including dmin chromosomes (*top panel*), duplications (*middle panel*), and different types of translocations (*bottom panel*). In all instances, chromosome translocations resulted in copy number increases of chromosome 11 sequences. Full karyotypes of all tumors can be viewed elsewhere.[9] *C–F*, high-resolution mapping of candidate genes involved in mammary gland tumorigenesis. Different from other murine models for breast cancer, deletions of neither *Trp53* nor *Brca1* are required for tumorigenesis in PyV-mT induced tumors, as both copies are present (*C*). The gain of chromosomes 11 and 15 suggested the amplification of the *HER2/Neu* (chromosome 11) and *Myc*-oncogenes (chromosome 15). However, FISH analysis revealed normal copy numbers for these genes (*D*). Likewise, two additional candidate genes, *Tbx2* and *Rad51c*, on the amplicon on chromosome 11 were not subject to copy number increases and did not label the dmin in the tumors (*E* and *F*). *G* and *H*, to determine more precisely the specific sequences amplified in the dmins, a BAC contig was assembled for chromosome band 11E2. Note that clone 284L12 is present in eight copies in tumor 404mt (*G*) and consistently labeled the dmins in tumor 158mt (*H*). The adjacent clone 382B19 (shown in *green*) was not involved. Clone 284L12 contains the *Sept9* gene. The insert shows the 4′,6-diamidino-2-phenylindole DNA counterstain of dmin chromosomes.

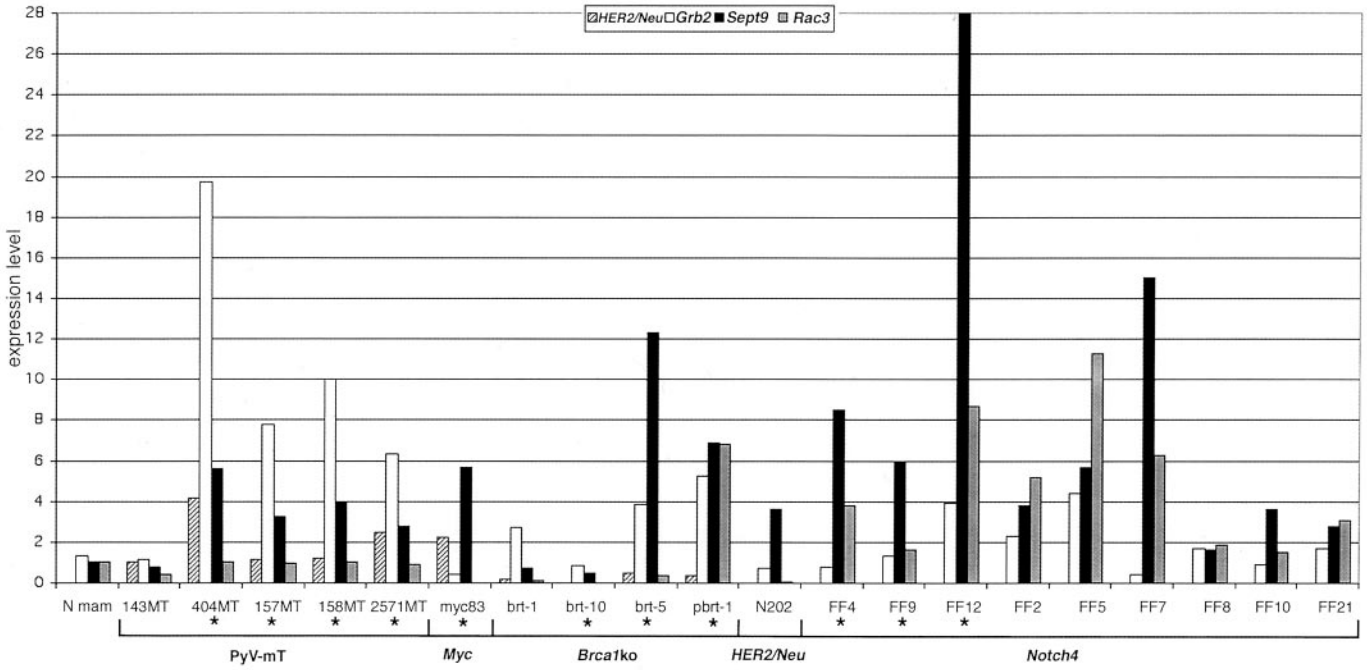

Fig. 3. Real-time PCR-based quantification of gene expression in the PyV-mT-induced tumors and in tumors arising by overexpression of c-*Myc*, *HER2/Neu*, or *Notch4*, and conditional knockout of *Brca1*. ∗ indicate the presence of ME2 genomic amplification. RNA extracted from normal mammary gland tissue and the cell line 143mt (which did not show genomic amplification of *Sept9*) were used as controls, and the standard expression level was assigned as 1. Note the significantly increased expression levels for *Sept9* in 16 tumors from the four mouse models analyzed. In contrast, overexpression of *Grb2* is only found in the PyV-mT tumors, whereas *Rac3* overexpression is observed occasionally in the *Notch4* tumors. With the exception of brt-10, where genomic amplification of 11E2 was not accompanied by increased expression of *Sept9*, copy number increases of chromosome band 11E2 were correlated to *Sept9* overexpression. In the *Notch4* transgenic mice we could detect increased transcription levels also in the absence of genomic amplification.

of cytogenetic abnormalities observed. In some tumors (157mt and 404mt), the BAC clones (RP23-284L12 and 84C12) containing sequence homology for *Sept9* and *Rac3* were present in additional copy numbers. In other tumors (158mt and 2571mt), only the *Sept9*-specific clone showed copy number increases. Of note, only this clone specifically labeled the dmins identified in a subset of tumors with gain on chromosome 11E2 (Fig. 2, *G* and *H*). Therefore, we conclude that the minimally amplified region comprises exclusively BAC clone RP23-284L12, the clone that contains the *Sept9* gene. Sequence analysis revealed that no other known genes are present in this genomic clone. It was important to determine whether the *Sept9* expression level was increased as a result of its genomic amplification. Therefore, we designed primers for quantitative real-time PCR. Because of the genomic coamplification of the *Rac3* gene in two tumors, primers specific for this gene were included in the analysis. Primers for the *Grb2* gene were included as a positive control, as this gene is specifically up-regulated by PyV-mT. The results showed that *Sept9*, but not *Rac3*, is consistently present at substantially increased expression levels in all of the cases with a genomic amplification of 11E2, when compared with the expression level in the normal mammary gland (Fig. 3). To assess whether *Sept9* gene amplification and overexpression is restricted to the PyV-mT model, we extended the transcription profiling to four additional models of human breast cancer where gain of chromosome 11 or amplification of the distal

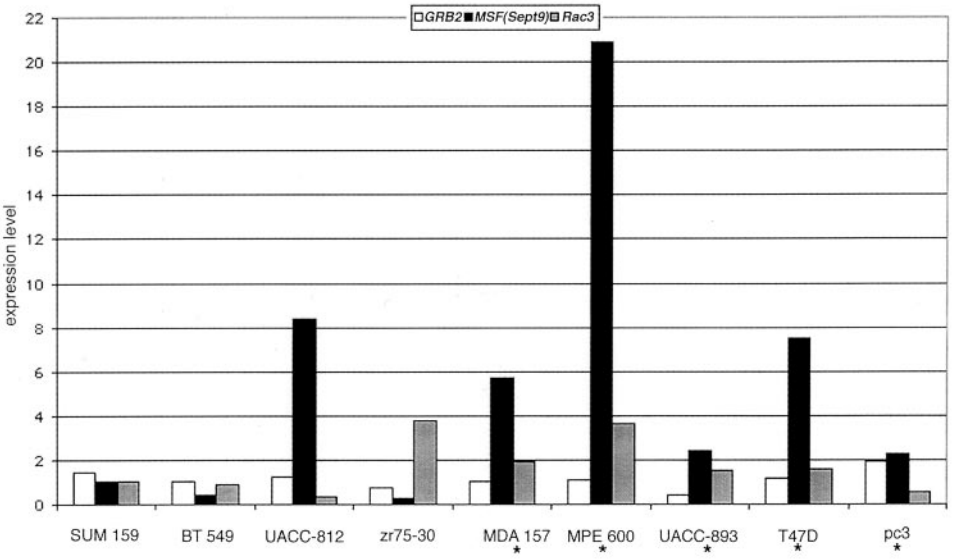

Fig. 4. Evaluation of *MSF (sept9)* expression levels in human breast carcinomas. The results of quantitative real-time PCR are plotted. Six of eight cell lines showed expression levels that ranged from 2.2-fold (UACC 893 and Pc3) to >20.8-fold (MPE 600). *MSF* (*sept9*) amplification also occurred independently of genomic copy number levels on human chromosome 17q25 (in tumor UACC-812), the chromosomal mapping position of the *MSF* (*sept9*) gene. *RAC3*, a gene that maps close to *MSF* (*sept9*) was not amplified consistently.

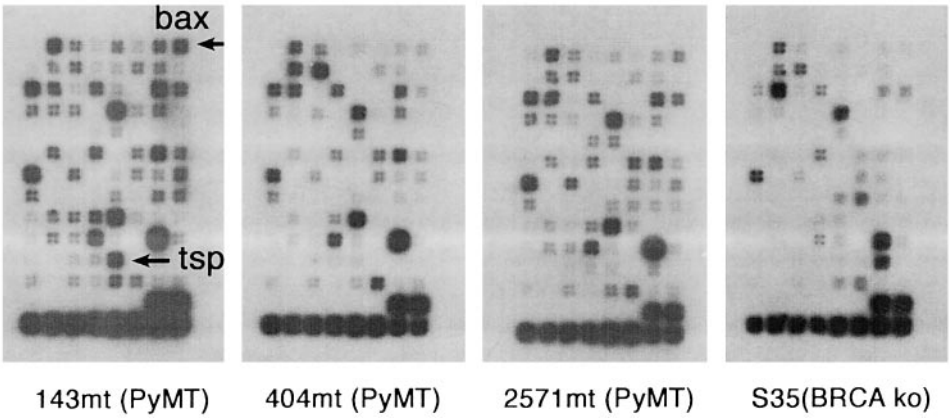

Fig. 5. Down-regulation of the apoptosis genes *Thsp1* and *Bax*. RNA from three PyV-mT tumors (143mt, 404mt, and 2571mt) and one *Brca1* conditional knockout (pbrt-5) was hybridized to a cDNA filter containing 96 genes involved in apoptosis. Tumors 404mt, 2571mt, and pbrt-5 have increased expression levels of *Sept9*; tumor 143mt has not. Note the down-regulation of *Bax* and *Thsp1* in the *Sept9*-positive tumors.

region of it are frequently observed. Myc 83, a tumor from a mouse model that overexpresses *c-Myc* under the control of the MMTV promoter, also revealed increased expression levels. *Sept9* levels were increased in two of four mammary gland adenocarcinomas from *Brca1* conditional knockouts as well. Additionally, we analyzed nine tumors from mice transgenic for *Notch4*. Increased transcript levels of *Sept9* defined the majority of the tumors, sometimes also in the absence of genomic amplification. Our results indicate that *Sept9* but not *Grb2* or *Rac 3* is the common target for mRNA overexpression in the region of genomic amplification. This suggests that cellular pathways that promote tumorigenesis independent of the oncogenic stimulus are dependent on, or at least benefit from, increased *Sept9* levels. Human chromosome 17q, which is orthologous to mouse chromosome 11, is gained frequently in human breast carcinomas. In fact, this chromosome arm contains several amplicons, with either known (*HER2/Neu* on chromosome band 17q13) or suspected oncogenes (18). However, in cytogenetic studies of human breast cancer we have also frequently observed increased copy numbers distal to chromosome band 17q23, as have other groups (19). The *MSF* (*Sept9*) gene maps to band 17q25. Therefore, we extracted RNA from a series of breast cancer cell lines, some of which showed gain or amplification on band 17q25 (20). The quantitative PCR clearly established that *MSF* (*Sept9*) is highly expressed in six of nine cell lines, and that the overexpression of this gene can occur in the absence of genomic copy number increase (Fig. 4). In two cell lines (MDA157, MPE600), *MSF* (*Sept9*) overexpression was accompanied by an increase in *RAC3* expression. *GRB2* mRNA levels were not increased. This finding establishes that abnormal expression of the *MSF* (*Sept9*) gene is not only involved in murine tumorigenesis but in human carcinomas as well.

Septins play a role in multiple cellular functions ranging from vesicle transport to cytokinesis. To identify in which cancer-related pathway the overexpression observed here exerts its function, we have used pathway finder array filters that allow one to query the expression levels of 96 genes simultaneously. One array contained genes involved in cell cycle regulation, and the second array contained genes that affect different cancer pathways. Four cell lines were tested, three of which were derived from the group of tumors induced by PyV-mT overexpression. One tumor, pbrt-5, was derived from conditional *Brca1* knockouts. The latter and two of the PyV-mT models showed 2.8–12.3-fold increased expression levels of *Sept9* (see Fig. 3). The two groups of tumors could be distinguished by the expression levels of *Thsp1* and *Bax*, which increased only in the absence of *Sept9* overexpression, *i.e.*, the one PyV-mT cell line that did not overexpress *Sept9* showed increased levels of *Thsp1* and *Bax* (Fig. 5). The down-regulation of *Thsp1* and *Bax* was confirmed in a larger series of tumors using quantitative reverse transcription-PCR (Fig. 6). We conclude that the down-regulation of *Thsp1* and *Bax* is linked to the overexpression of *Sept9*. Up-regulation of both *Thsp1* and *Bax* induces apoptosis (21, 22).

In addition to pathway dissection, another way of performing functional analysis of a gene is to inhibit its expression using interference RNA. To determine the consequences of *Sept9* gene inactivation we designed siRNAs and transiently transfected cells with and without *Sept9* overexpression. Successful transfection was determined by real-time PCR as the levels of detectable RNA decreased. mRNA expression decrease (28.8–58%) was observed in the three cell lines with *Sept9* overexpression. Independent of that, we determined transfection efficiency using GFP siRNA on the same cells in combination with a GFP vector. Whereas GFP transfection alone revealed 40% positive cells, none of the cells showed green fluorescence when the GFP vector was combined with siGFP, indicating the successful transfection. Under the conditions used here, the inactivation of *Sept9* expression induced no visible morphological changes, or changes on the growth kinetics of the cell populations, nor was DNA synthesis impaired as measured by bromodeoxyuridine incorporation. However, reduction of *Sept9* expression by siRNA resulted in the up-regulation of *Bax* message in cell lines 404mt, 2571mt, and brt-5, whereas the expression levels in the cell line that did not overexpress *Sept9* were reduced slightly (Fig. 7*A*). Treatment of the cells with *Sept9* siRNA also caused the up-regulation of *Thsp1* in cell lines 404mt, 2571mt, and brt-5, all three belonging to the *Sept9*-overexpressing group (see Fig. 7*B*). *Thsp1* levels were down-regulated in cell line 143mt, which showed no *a priori* increase of *Sept9* levels (Fig. 7*B*).

DISCUSSION

Here we report the molecular cytogenetic characterization of 26 tumor samples derived by overexpression of the PyV-mT under the control of the MMTV promoter. As in other mouse models of human breast cancer, tumor formation is accompanied by, or requires, the acquisition of specific chromosomal aberrations. The vast majority of these aberrations result in genomic gains or losses. It has been firmly established that chromosomal regions of recurrent genomic imbalance point to the location of tumor suppressor genes in case of chromosomal loss or to oncogenes when genomic amplification is observed. Therefore, we were intrigued by the consistent copy number increase on mouse chromosome 11. Mouse chromosome 11 contains sequences orthologous to human chromosomes 22, 16, 5, and 17 (23).[10] In fact, most of human chromosome 17 (HSA17) resides on mouse

[10] Internet address: http://www.ncbi.nlm.nih.gov/Homology/index.html.

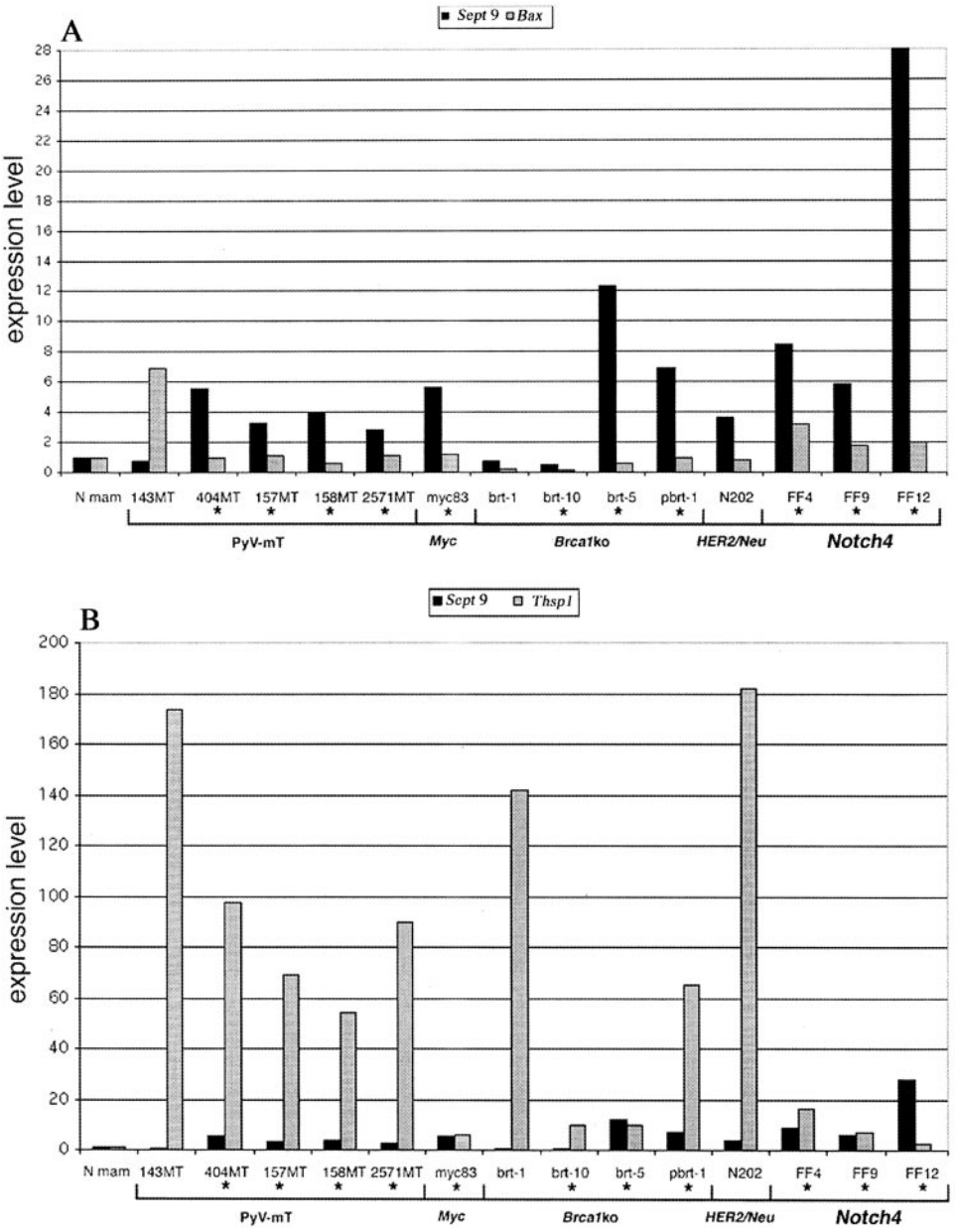

Fig. 6. Quantitative real-time PCR analysis of *Bax* (*A*) and *Thsp1* (*B*) levels as compared with *Sept9* expression levels. The tumor number and induction mode is provided below the graphs. The ∗ denote those tumors in which genomic amplification of *Sept9* was observed. Note that increased expression levels of *Sept9* correlate with reduced expression levels of *Bax* and *Tshp1* in the majority of the tumors, independent of mode of tumor induction.

chromosome 11 (MMU11). Many genes known to be involved in breast cancer map to this chromosome, including *Trp53*, *HER2/Neu*, and *Brca1*. In addition, CGH analyses of human breast cancers identified another region of frequent amplification that maps to chromosome band 17q23 (16, 17). Candidate genes in this region include *Rad51c* and *Tbx2*. Therefore, the significance of this chromosome prompted us to identify the critical region with higher resolution. On the basis of available sequence maps, we selected a set of BAC clones for high-resolution FISH mapping. This effort was greatly aided by the fact that in two of the tumors with amplification on chromosome 11, dmins were present in metaphase spreads. One of the BAC clones, RP23-284L12, labeled the dmin in two tumors (158mt and 2571mt) and translocated fragments of chromosome 11 in two additional ones (404mt and 157mt). Adjacent BAC clones, both distal and proximal of 284L12, labeled only the normal copies of chromosome 11 but not the dmin copies. In this regard, we were particularly interested in whether the amplicon on chromosome 11 contained genomic sequences for *Grb2*. Overexpression of *Grb2* expedites tumor development in PyV-mT mice. A high level of *GRB2* mRNA is also observed in human breast cancer cell lines, accompanied in some instances by genomic amplification (24, 25). The real-time PCR results showed clearly that *Grb2* expression is elevated in most of the PyV-mT-derived tumor cell lines. However, the BAC clone containing the murine *Grb2* gene was not amplified and did not label the dmins nor did it label the extra fragments of chromosome 11 involved in copy number increases. Therefore, we conclude that the genomic amplification on chromosome band 11E2 and the presence of dmins is not driven by the requirement for increased message of *Grb2*, but that *Grb2* expression is up-regulated by mechanisms other than genomic amplification. Therefore, *Sept9* is the only known gene in the minimal overlapping region of genomic amplification on mouse chromosome 11. One tumor, brt-10, which was derived from *Brca1* conditional knockout mutants, revealed genomic amplification of MMU11 band, which was not accompanied by increased expression levels of *Sept9*. This could be because the genomic amplicon actually does not contain *Sept9*, a possibility that is likely because the CGH ratio profile

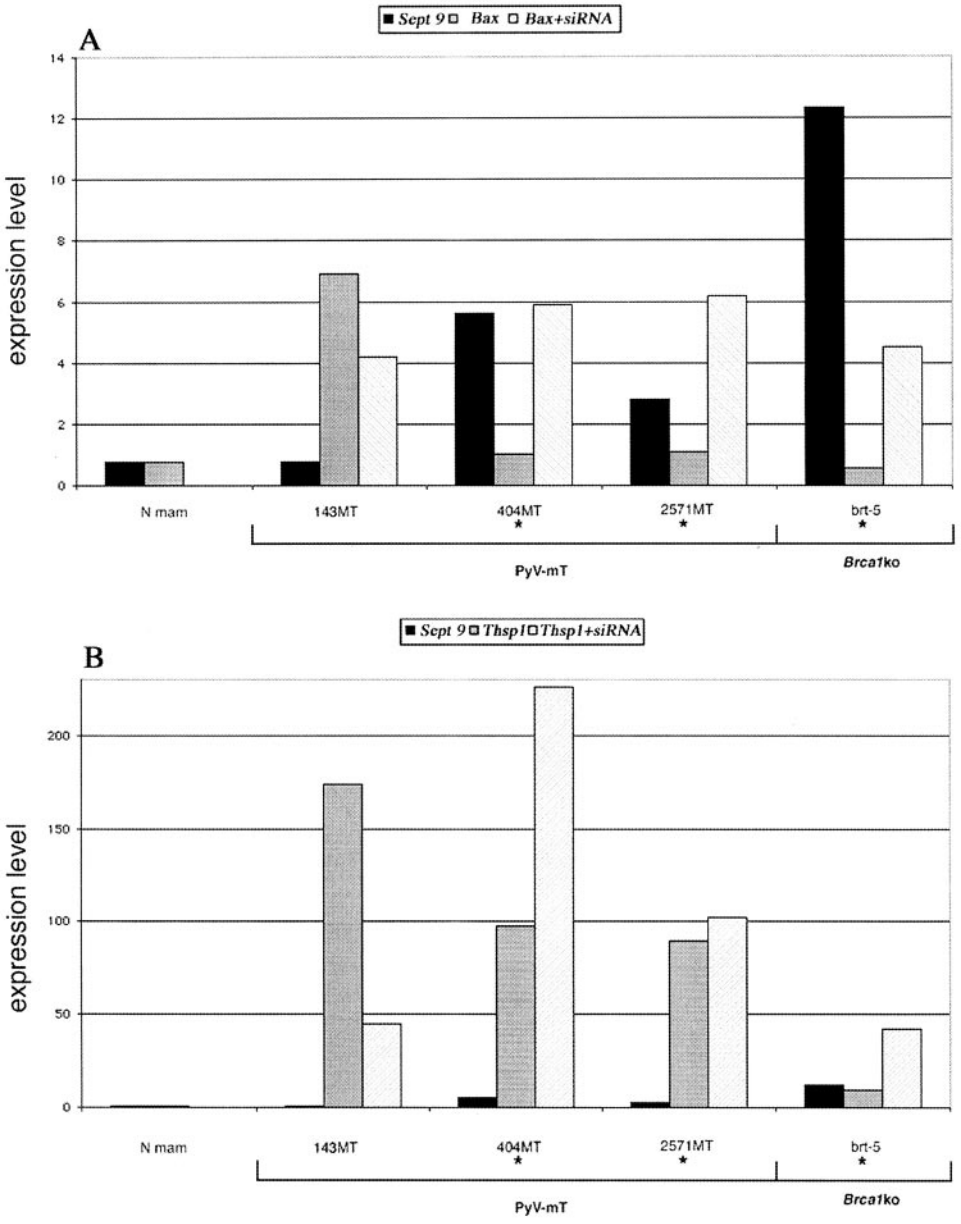

Fig. 7. Consequences of *Sept9* inhibition by siRNA on the expression levels of the apoptosis genes *Bax* and *Thsp1*. The tumor numbers are provided below the graphs. The * denote those tumors in which genomic amplification of *Sept9* was observed. ■ indicate the expression levels of *Sept9* before siRNA was added to the medium. In all tumors that showed increased *Sept9* expression levels, siRNA resulted in a pronounced up-regulation of both *Bax* and *Thsp1*.

suggests that *Sept9* is not included in the amplicon in this particular case (data not shown). *Sept9* overexpression is not restricted to the PyV-mT model. In fact, even higher levels of *Sept9* were found in MMTV-*myc* and MMTV-*Notch 4* tumors, and in tumors arising in conditional mutants for *Brca1*. This and the observation that increased *Sept9* levels were also present in the majority of human breast cancer cell lines included in this study suggest that this gene is involved rather ubiquitously in tumorigenesis in the mammary gland. Although tumorigenesis arises through different initial pathways in these models, the resulting overexpression of the same gene argues in favor of its importance in a downstream pathway.

In yeast, members of the septin family are required for mitosis-specific activation of the Gin4 kinase, and the loss of septin causes a mitotic delay (26). In *Drosophila,* mutations in the septin genes, such as *sep1* and *pnut*, cause the formation of multinucleated cells (27). The septin family is conserved in humans. One human septin, NEDD5, localizes to the cleavage furrow, and functional inactivation of this septin by antibody injection results in the disruption of cytokinesis (28). Therefore, it appears that one role of the septins relates to the successful completion of mitosis, a function that is conserved from yeast to human. In addition to this function, septins contain a conserved domain with GTPase activity (29).

Several papers have reported that *MSF* (*Sept9*) maps to a chromosomal region that is consistently lost in ovarian carcinomas (30, 31). This led to the hypothesis that septin may function as a tumor suppressor gene in breast and ovarian carcinomas. This interpretation is not supported by our findings, because genomic gain and amplification of the *MSF* (*Sept9*) are observed consistently in quite different mammary gland adenocarcinomas and in cell lines established from human breast tumors. Most importantly, the genomic amplification is invariably accompanied by the overexpression of this gene in the tumors analyzed here. In some tumors, overexpression is observed even in the absence of genomic amplification. Also, the presence of this gene in the dmins strongly argues for its role as an oncogene rather than in tumor suppression. This notion is additionally corroborated by the results published by Risk *et al.* (32), who actually excluded *MSF* (*Sept9*) from a commonly deleted region on human chromosome 17q25.

Translocation of two members of the septin genes to the *MLL* gene on chromosome 11q23 in acute myeloid leukemia has been reported from two groups (33, 34). Whereas evidence has not yet been established unambiguously, it seems that *MLL* alone is not sufficient for leukemogenesis. This is supported by the fact that these chromosomal translocations occur in frame, hence producing a fusion protein. This is entirely consistent with the paradigm of chromosomal translocations in leukemia, including the *BCR-ABL* fusion as a consequence of the Philadelphia chromosome (35). Therefore, it seems reasonable to assume that septins, when activated or overexpressed, provide a growth advantage to cells, in particular to rapidly growing cancer cells.

The pathway blots and quantitative reverse transcription-PCR analyses that we have used to query how increased *Sept9* levels interact in the signaling cascades in mammary tumor formation suggest that two proteins that mediate apoptotic response were selectively down-regulated as a consequence of *Sept9* overexpression. In an attempt to understand the consequences of increased cellular septin levels in breast cancer cells, we used interference RNA to experimentally reduce septin levels in three cell lines where we observed overexpression as well as in a control one that revealed normal expression. Transfection with siRNA resulted in increased expression levels of *Thsp1* and *Bax*, and in all of the tumors with elevated *Sept9* levels, but not in the tumors without genomic amplification and overexpression of *Sept9*, additionally corroborating the functional significance of the genetic alteration. However, none of the investigated parameters that we assessed, such as morphology, DNA synthesis, or population doubling times were altered significantly. Whereas at the moment we are lacking a clear explanation for this phenomenon, we hypothesize that either the transfection efficiency of ~40% was too low to affect the cancer cell population as a whole or that redundant pathways that rescue septin deficiency compensate for *Sept9* down-regulation.

In summary, we provide evidence for a generalized *Sept9* overexpression in multiple and diverse mouse models of human breast cancer and in human breast cancer cell lines. On the basis of their known roles in cytokinesis and control of mitotic cell division, we suggest that increased septin levels might assist in the rapid mitotic turnover that is characteristic for malignant cells. This could be facilitated by the observed inhibition of *Thsp1*- and *Bax*-triggered apoptotic response.

ACKNOWLEDGMENTS

We thank Buddy Chen for manuscript editing, Dr. Vittorio Sartorelli for advice on the design of the siRNA experiments, and Dr. Stan Lipkowitz for valuable discussion and comments.

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